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Duplex-specific nuclease assisted miRNA assay based on gold and silver nanoparticles co-decorated on electrode interface

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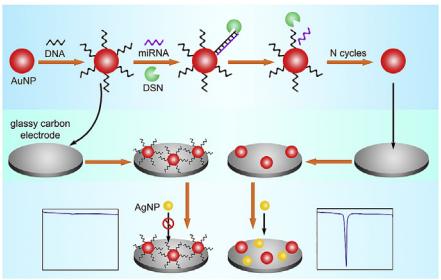
HIGHLIGHTS

- A highly sensitive electrochemical biosensor for the detection of miRNA is developed.
- The interaction between DNA modified gold nanoparticles and silver nanoparticles is controlled by miRNA triggered reaction.
- The biosensor successfully distinguishes targets from interfering miRNAs.
- The biosensor performs satisfactorily in real biological samples.

GRAPHICAL ABSTRACT



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ABSTRACT

miRNAs are small non-coding RNAs for gene regulation, which serve as promising biomarkers for the diagnosis of certain diseases. In this contribution, we have proposed a convenient electrochemical biosensing strategy based on the interaction between DNA modified gold nanoparticles (AuNPs) and silver nanoparticles (AgNPs). In principle, citrate capped AuNPs and AgNPs can be co-decorated on the electrode successively. However, with the modification of DNA on AuNPs surface, a strong negative layer is formed. AuNPs@DNA modified electrode could then inhibit subsequent adsorption of AgNPs due to the electrostatic repulsion and steric hindrance effect. As a result, electrochemical response from AgNPs is significantly decreased. On the other hand, in the presence of target miRNA, DNA on AuNPs hybridizes with miRNA and can thus be cyclically digested by duplex-specific nuclease (DSN). Without the shield of DNA, AgNPs can be relaunched at the AuNPs modified electrode. By analyzing the silver stripping peak, highly sensitive detection of miRNA can be achieved. This biosensor exhibits the limit of detection as low as 0.62 fM and a broad linear range from 1 fM to 1 pM. It may hold great potential utility for miRNA assay in the applications of biomedical researches and early clinical diagnosis.

1. Introduction

miRNAs are a type of endogenous non-coding RNAs, which consist of 18–25 nucleotides [1]. They exist in most of living organisms and play a critical role in many biological events such as

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cell proliferation, differentiation, apoptosis, and so on [2–4]. Aberrant expression of miRNAs may cause uncontrolled gene regulation and interfere normal cellular pathways [5]. Accumulative evidences have indicated that the miRNA expression is closed related with many diseases including cancers [6,7]. Thereby, specific miRNA can be considered as promising disease markers for early diagnosis [8,9]. For instance, many cancer associated miRNAs have been detected in the samples of blood serum, saliva, and urine, which provide important information about diseases and certain pathways [10–12]. Nevertheless, there are certain difficulties for convenient and highly sensitive analysis of miRNA [13]. First, miRNA only accounts for a small fraction of total RNA; second, the short length makes it much more difficult for quantification compared with longer RNAs; third, sequence homology between miRNA family members may result in severe false positive results in the assay. Thus, it is imperative to develop accurate, selective and convenient sensing strategies for miRNA detection [14,15]. Recently, a number of methods, especially electrochemical methods, have been developed as alternatives to traditional qRT-PCR technique [16,17]. For example, Gai et al. entrapped electrochemical signals in the pores of mesoporous silica nanoparticle which could be released by miRNA triggered reaction. Besides, another electroactive probe was introduced to facilitate redox recycling and to improve the sensitivity for miRNA assay [18]; Shi et al. constructed DNA nanoflowers to provide amplified steric hindrance charge in nanochannels, which could be used to reflect the level of initial target miRNA [19]; Xue et al. synthesized CeO_2 nanospheres co-doped with Cu and Co as efficient electrocatalysts for dual-mode electrochemical sensing of miRNA [20].

To make theoretical miRNA assays more practical, robust materials and moderate reaction steps are usually favorites. In this work, we have proposed a simple but highly sensitive electrochemical method for miRNA detection. Widely used silver nanoparticles (AgNPs) are applied as the electrochemical species. After adsorbed on the glassy carbon electrode, AgNPs provide sharp and intense silver stripping peak [21]. However, in the presence of pre-immobilized DNA modified gold nanoparticles (AuNPs), due to the effects of electrostatic repulsion and steric hindrance, AgNPs cannot be located on the electrode interface and the electrochemical response is shut down. In the presence of target miRNA, specific hybridization event occurs between miRNA and DNA on the surface of AuNPs. Duplex-specific nuclease (DSN) mediated digestion and target recycling facilitate the elimination of DNA [22]. As a result, the pre-immobilized AuNPs cannot repel AgNPs and the two nanoparticles are co-decorated on the surface of the electrode. By analyzing the increased electrochemical response, target miRNA level can be determined. This method achieves high sensitivity and selectivity. Remarkably, it only involves the modification of commonly used enzyme and nanomaterials, which makes it suitable for practical applications.

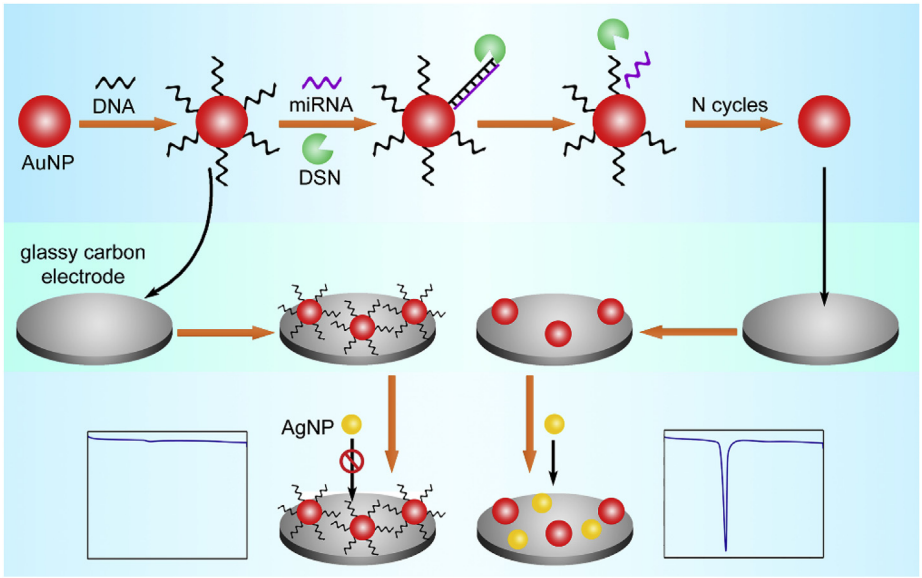
2. Experimental

2.1. Materials and chemicals

Chloroauric acid ($HAuCl_4$), silver nitrate ($AgNO_3$), sodium borohydride ($NaBH_4$), sodium hydroxide (NaOH), trisodium citrate, citric acid, diethypyrocarbonate (DEPC), tris(2-carboxyethyl)phosphinehydrochloride(TCEP), poly(diallyldimethylammonium chloride) (PDDA), and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma (St. Louis, MO). All other chemicals were of analytical grade and used without further treatment. DSN was ordered from Genomax Technologies Pte Ltd. (Singapore). One Step qRT-PCR Kit was from Tiangen Biotech Co., Ltd. (Beijing, China). GeneRuler Ultra Low Range DNA Ladder was purchased from Thermo Fisher Scientific Inc. (United States). DNA and RNA probes were synthesized and purified by Takara Biotechnology Co., Ltd. (Dalian, China). The sequences were listed in Table S1. Double-distilled water purified by a Millipore water purification system with a specific resistance of 18 MΩ cm was further treated with DEPC (1‰, v/v) before used to prepare all solutions.

2.2. Preparation of AuNPs and AgNPs

Bare AuNPs were synthesized by citrate reduction of $HAuCl_4$ according to the literature [23]. Briefly, 5 mL of trisodium citrate (38.8 mM) was quickly added to 50 mL of $HAuCl_4$ refluxing solution 0.01%(w/v) under stirring and boiling for 15 min. After stirring for another 30 min to ensure complete reaction, the solution was cooled down to room temperature slowly. DNA modification on the surface of AuNPs was achieved by the well-known gold-sulfur chemistry [24]. Thiolated DNA strand was added into bare AuNPs



Scheme 1. Illustration of the electrochemical method for the detection of miRNA.

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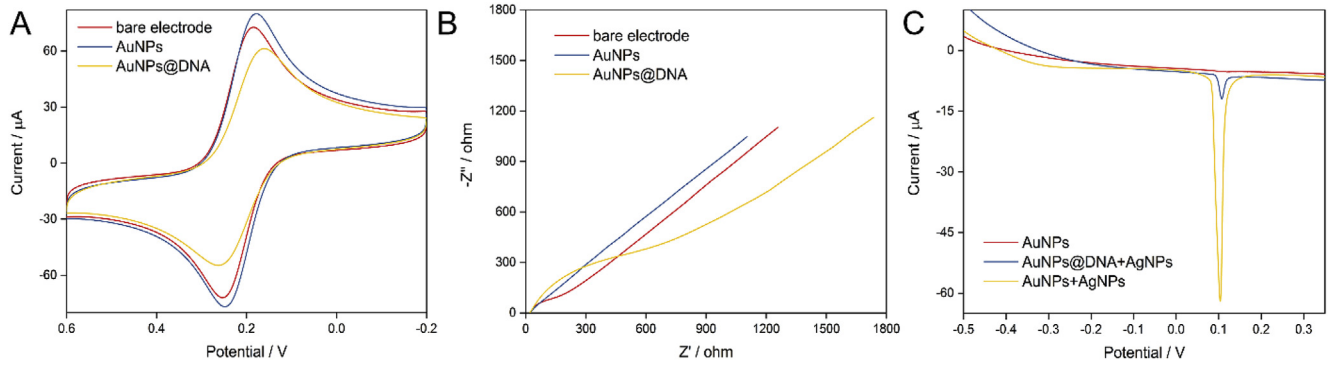

Fig. 1. (A) Cyclic voltammograms and (B) nyquist diagrams of bare electrode, after the treatments with AuNPs and AuNPs@DNA, respectively. (C) Linear sweep voltammograms of the modified electrode before and after the incubation with AgNPs.

solution. The final concentrations of DNA and AuNPs were 3 μM and 10 nM, respectively. The ratio was 300 : 1. pH was then adjusted to 3.0 with citrate buffer, which was determined by pH test strips. Afterward, NaOH solution (2 M) was added into the mixture dropwise until pH was increased to 7.4. The resulted solution was purified by centrifugation at 13000 rpm for 30 min. The red precipitate was washed by pure water, centrifuged, and dispersed in pure water. AgNPs were synthesized by the borohydride reduction of $AgNO_3$ [25]. In short, the solution containing 0.25 mM $AgNO_3$ and trisodium citrate was prepared. Next, 10 mM $NaBH_4$ solution was prepared. 100 mL of Ag^+ solution was blended with 3 mL of $NaBH_4$ solution, and the mixture was under violent stirring for 30 min. After rest overnight, the yellow colored solution was purified by centrifugation at 12000 rpm for 30 min.

2.3. Pretreatment of glassy carbon electrode

Glassy carbon electrode was firstly incubated with piranha solution (98% H_2SO_4: 30% H_2O_2 = 3:1) for 5 min in order to remove any adsorbed materials. (*Caution: piranha solution dangerously attacks organic matter!*) Subsequently, it was rinsed with double-distilled water and polished with P3000 silicon carbide paper until a mirror-like surface was obtained. The electrode was further sonicated in ethanol and then water, each for 5 min. Afterward, it was dried with nitrogen before further use.

2.4. Quantification of target miRNA

A series of standard miRNA solutions were firstly prepared. The AuNPs@DNA were mixed with different amount of miRNA and treated with 1.0 U DSN at 50 °C for 100 min for cleavage reaction. After that, AuNPs were purified by centrifugation at 13000 rpm for 30 min. The pretreated glassy carbon electrode was incubated with 10 μL of 4.0 g L^{-1} PDDA solution (containing 0.05 M NaCl) for 0.5 h. This attached positively charged precursor layer benefitted further adsorption of negatively charged nanoparticles. After careful rinsing, the electrode was immersed in the obtained AuNPs (300 μL) for 1 h [26]. Next, it was rinsed and further treated with AgNPs (300 μL) for another 0.5 h.

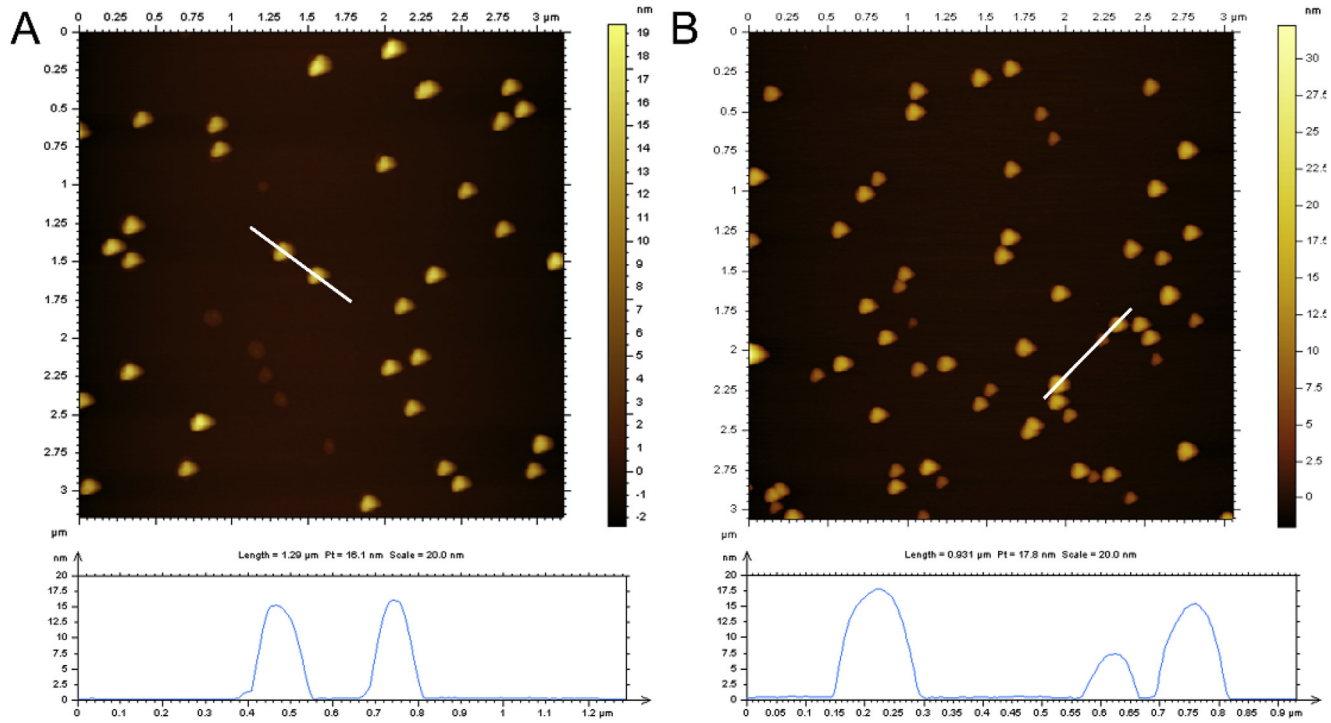

Fig. 2. AFM images of DSN treated (A) AuNPs@DNA, (B) mixture of AuNPs@DNA and miRNA before further incubation with AgNPs.

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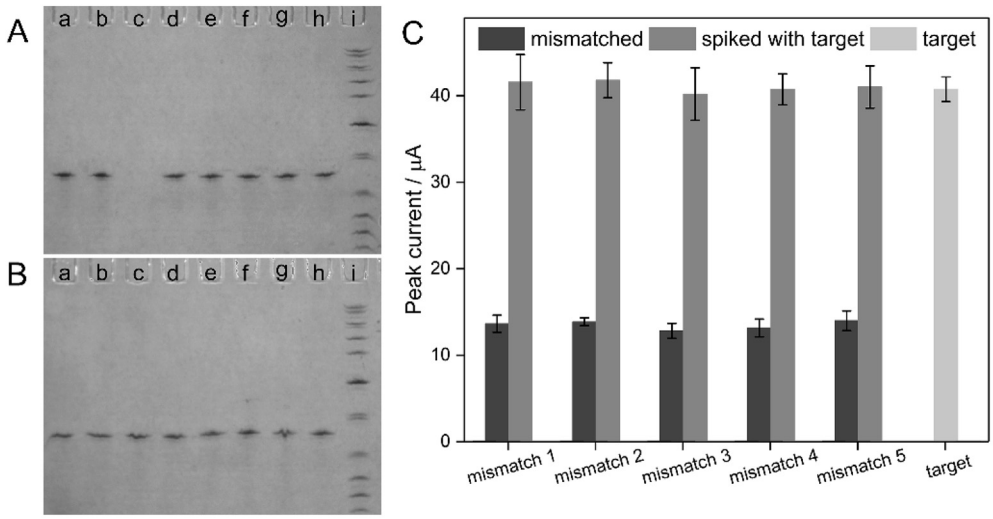

Fig. 3. Polyacrylamide gel electrophoresis analysis of (A) cDNA and (B) rDNA: (a) DNA; (b) DNA and DSN; (c) DNA, miR-106a, and DSN; (d) DNA, mismatch 1, and DSN; (e) DNA, mismatch 2, and DSN; (f) DNA, mismatch 3, and DSN; (g) DNA, mismatch 4, and DSN; (h) DNA, mismatch 5, and DSN; (i) GeneRuler Ultra Low Range DNA Ladder. (C) Comparison of LSV peak currents for the detection of interfering miRNAs with and without the addition of target miRNA.

2.5. Electrochemical measurements

All electrochemical experiments were carried out on a CHI 660D electrochemical workstation (CHI instruments, China). The three electrode system was applied, including a platinum wire auxiliary electrode, an Ag/AgCl reference electrode and the glassy carbon working electrode. Linear sweep voltammetry (LSV) experiments were performed in the electrolyte of 0.1 M KCl with the scan rate of 0.1 V s^{-1}. Cyclic voltammetry (CV) was performed with the scan rate of 0.6 to −0.2 V and the scan rate of 50 mV s^{-1}. Electrochemical impedance spectroscopy (EIS) was carried out with the parameters as follows: biasing potential, 0.22 V; amplitude, 5 mV; frequency range, 0.1 Hz to 100 kHz.

3. Results and discussion

3.1. Biosensing principle

Scheme 1 illustrates the detailed working principle. Generally, citrate capped AuNPs and AgNPs are firstly prepared. AuNPs are further modified with the complementary DNA (cDNA) of target miRNA via gold-sulfur chemistry. In the presence of miRNA, DNA/RNA duplex forms, which can be recognized by DSN. This enzyme specially digests DNA strand in DNA/RNA duplex [27]. Therefore, miRNA is released, which can be used to trigger new cycles of DSN cleavage reactions. As a result, a number of DNA strands on AuNPs can be removed. After immobilized on the surface of electrode, AgNPs can be further loaded by inserting in the intervals between AuNPs. Since a highly characteristic solid-state Ag/AgCl reaction occurs, intense silver stripping peak can be obtained. In addition, AuNPs with excellent electrical conductivity are helpful for the improvement of electrochemical response. On the other hand, in the absence of miRNA, DNA strands remain on the surface of AuNPs. The negatively charged DNA strands inhibit the adsorption of citrate capped AgNPs by electrostatic repulsion, which can also be enhanced by the steric hindrance effect of DNA [28–30]. Therefore, the silver stripping peak is decreased significantly. By comparing the LSV peak intensities, the initial miRNA concentration can be evaluated.

3.2. Qualitative experiments

The synthesized AuNPs and AgNPs are characterized by transmission electron microscopy, from which the diameters about 15 and 6 nm are determined, respectively (Figure S1). Successful immobilization of AuNPs and AuNPs@DNA on the electrode can be confirmed by CV and EIS. As depicted in Fig. 1A, a pair of well-defined current peaks is observed for bare gold electrode. After modified with AuNPs, due to the excellent electrical conductivity of the nanoparticles, the peak currents increase. However, with the case of AuNPs@DNA, the negatively charged DNA is able to repel $[Fe(CN)_6]^{3-/4-}$, leading to the drop of the peaks. CV results are in good accordance with nyquist diagrams in Fig. 1B. The diameter of the semicircle domain of the diagram represents charge transfer resistance. After modified with bare AuNPs, the domain gets smaller and R_{ct} decreases from 96.61 to 30.22 Ω. On the contrary, AuNPs@DNA modification increases the diameter due to the negatively charged interface caused by DNA. R_{ct} increases to 242.5 Ω. The subsequent loading of AgNPs is investigated by LSV. As shown in Fig. 1C, no stripping peak can be observed at AuNPs modified electrode. After incubating with AgNPs, since there is no effective electrostatic repulsion and steric hindrance, AgNPs can be easily decorated in the intervals between AuNPs. A significant LSV peak appears. However, with AuNPs@DNA modified electrode, the immobilization of AgNPs can be effectively inhibited, which is reflected by the tiny LSV peak. We have then taken atomic force microscopic (AFM) images to further characterize the immobilization interfaces. In the absence of target miRNA, DSN cannot function and DNA remains on the surface of AuNPs, which hinders the adsorption of AgNPs. As shown in Fig. 2A, only 15 nm sized AuNPs can be observed in the AFM image. On the contrary, DNA strands can be digested after introduction of target miRNA, which initiates DSN reaction and facilitates the localization of AgNPs. In the corresponding AFM image, smaller nanoparticles with the diameter are ascribed to AgNPs, which is the direct evidence of the miRNA initiated reaction (Fig. 2B).

3.3. Selectivity investigation

To demonstrate that DSN catalyzed digestion cycles can be triggered only in the presence of target miRNA, we have introduced

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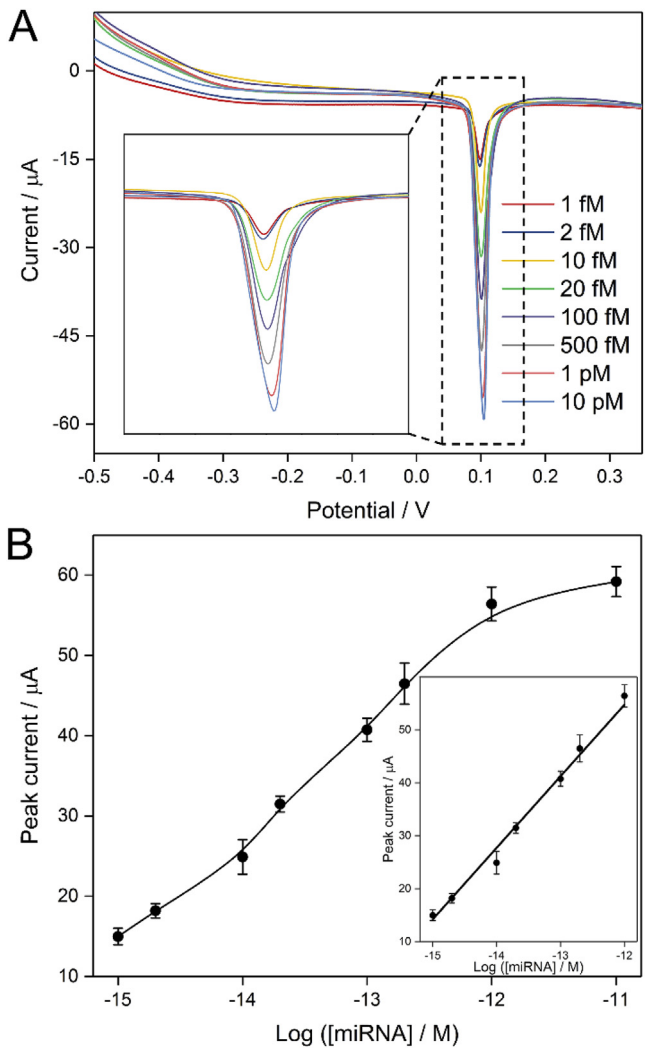

Fig. 4. (A) Linear sweep voltammograms for the detection of miRNA with the concentration of 1 fM, 2 fM, 10 fM, 20 fM, 100 fM 500 fM, 1 pM and 10 pM (from up to bottom). (B) Calibration plot of the LSV peak current versus the logarithmic concentration of miRNA. Inset shows the linear range. Error bars represent standard deviations of three independent measurements.

five miRNAs with one or more mismatch sites. As shown in Fig. 3A, cDNA cannot be cleaved by DSN directly. After hybridization with target miRNA with a much smaller concentration, the band of cDNA disappears, confirming the enzyme activity of DSN. However, after introducing other miRNAs, since they cannot form DNA/RNA duplex with cDNA, the bands remain in the gel image, indicating that these miRNAs cannot trigger DSN cleavage cycles. We have then replaced cDNA by a new DNA strand with random sequence (rDNA). As depicted in Fig. 3B, the DNA cannot be cleaved in the absence or presence of various miRNAs since no DNA/RNA duplexes can be formed. Next, we have applied LSV responses in the presence of various miRNA sequences. As shown in Fig. 3C, only with target miRNA initiated reaction, AgNPs can be decorated on electrode, which is reflected by the significant electrochemical responses. These results demonstrate excellent selectivity of the method towards target miRNA.

3.4. Quantification of miRNA

The analytical performances of this biosensor can be potentially influenced by immobilization of AgNPs and the process of DSN reaction. Thus, different reaction times are investigated. With longer incubation time of AgNPs in the case of bare AuNPs modified electrode, the peak intensity increases and reach a plateau in 30 min. In addition, more complete reaction of DSN may digest more DNA strands to eliminate the inhibition of AgNPs adsorption. After reacting for 100 min, the increase of peak current is retarded. Therefore, the reaction times of 30 min (AgNPs) and 100 min (DSN) are applied, respectively (Figure S2). Under optimal experimental conditions, the biosensor is used to quantify miRNA with a series of concentrations. Corresponding LSV peaks are displayed in Fig. 4A. With the increase of target miRNA from 1 fM to 10 pM, the peak current increases. The detailed relationship between peak current and logarithmic concentration of miRNA is shown in Fig. 4B. A good linearity is established from 1 fM to 1 pM with the R^2 of 0.994. The fitting equation is $y = 216.85 + 13.51\,x\,(n = 3)$, in which y stands for the peak current and x is the logarithmic concentration of miRNA. The limit of detection (LOD) is calculated to be 0.62 fM, which is quite low. A comparison with other recent miRNA biosensors using different techniques are listed in Table 1. The LOD of this biosensor is better or at least similar compared with other methods. The high sensitivity benefits from the following aspects. First, DSN mediated digestion reaction releases target and the recycling is an important amplification process. Second, AgNPs are excellent electrochemical nanoprobes, which provide intense silver stripping peaks. Third, AuNPs with excellent electrical conductivity act as the enhancer of electrochemical responses. In addition, this method avoids the use of expensive instruments/reagents and reliable analysis of miRNA can be achieved for potential practical use.

3.5. Detection of miRNA in biological samples

Serum has similar chemical components to blood plasma and is suitable to be used for disease diagnosis. Thus, the proposed biosensor is challenged by serum samples. These samples are collected from local hospital, which are firstly spiked with miRNA (10, 20, and 100 pM). After that, they are tested by the biosensor and qRT-PCR kits. The results are calculated and compared, which

Table 1
Comparison of analytical performances of the proposed method with recently developed miRNA assays.

Technique	Strategy	Detection range (M)	LOD (M)	Ref.
fluorescence	competitive DNA strand displacement	2×10^{-10} to 2.5×10^{-7}	10^{-12}	[31]
fluorescence polarization	T7 exonuclease and streptavidin coated nanospheres	10^{-12} to 10^{-8}	10^{-12}	[32]
lateral flow assay	catalytic hairpin assembly on AuNPs	10^{-11} to 10^{-6}	8.9×10^{-13}	[33]
SERS	oriented assembly of AuNPs	10^{-12} to 10^{-9}	1.2×10^{-13}	[34]
fluorescence	cycling click chemical ligation-illuminated fluorescent magnetic nanoparticles	10^{-13} to 5×10^{-11}	5×10^{-14}	[35]
amperometry	DNA tetrahedral nanostructure probe	10^{-14} to 10^{-9}	10^{-14}	[36]
SERS	mismatched catalytic hairpin assembly	10^{-14} to 10^{-7}	3.5×10^{-15}	[37]
fluorescence	graphene oxide based isothermal exponential amplification	10^{-14} to 10^{-11}	3×10^{-15}	[38]
fluorescence	target recycling and rolling circle amplification	10^{-15} to 10^{-12}	3.5×10^{-16}	[39]
LSV	DSN, AuNPs and AgNPs	10^{-15} to 10^{-12}	6.2×10^{-16}	this method

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are quite consistent (Table S2). The recoveries are from 92.80% to 107.05%, indicating that the biological samples do not interfere accurate detection of target miRNA.

4. Conclusions

In summary, a novel electrochemical biosensor has been successfully fabricated for the analysis of miRNA based on the co-decoration of AuNPs and AgNPs on the electrode interface. Taking advantages of DSN catalyzed target recycling amplification and strong electrochemical response of AgNPs, high sensitivity and selectivity are achieved. Real application for the analysis of serum samples shows great practical utility. The method shows the merits of cost-effective, rapid and stable detection. It might be applied for the miRNA related biomedical researches and screening of miRNA biomarkers for diseases diagnosis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Mingyuan Wang: Investigation, Data curation, Writing - original draft. **Wei Chen:** Data curation, Writing - original draft. **Longhai Tang:** Formal analysis, Data curation. **Ruhong Yan:** Validation, Writing - review & editing. **Peng Miao:** Conceptualization, Supervision, Writing - review & editing, Project administration.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2020.01.041.

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